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Leemhuis, H; Dijkhuizen, L

Published in:
Biocatalysis and Biotransformation

DOI:
10.1080/10242420310001614333

Publication date:
2003

Biocatalysis and Biotransformation, 2003 VOL. 21 (4/5). pp. 261 – 270

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Engineering of Hydrolysis Reaction Specificity in the Transglycosylase Cyclodextrin Glycosyltransferase

HANS LEEMHUIS and LUBBERT DIJKHUIZEN*

Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

(Received for publication 6 April 2003; Accepted for publication 23 May 2003)

Cyclodextrin glycosyltransferase (CGTase) is a member of the α-amylase family, a large group of enzymes that act on α-glycosidic bonds in starch and related compounds. Over twenty different reaction and product specificities have been found in this family. Although three-dimensional structure elucidation and the biochemical characterization of site-directed mutants have yielded a detailed insight into the mechanism of bond cleavage, the variation in reaction and product specificity is far from understood. This article gives an overview of recent developments in the understanding and engineering of transglycosylation and hydrolysis specificity in CGTase, which is one of the best-studied α-amylase family enzymes.

Keywords: Alpha-amylase; Carbohydrate; CGTase; Cyclodextrin; Hydrolase; Starch

Abbreviations: CGTase; cyclodextrin glycosyltransferase; BC251; *Bacillus circulans* strain 251.

INTRODUCTION

Cyclodextrin glycosyltransferase (CGTase) is a member of the α-amylase family (Janecek, 1997; Svensson, 1994; Takata *et al*., 1992), glycoside hydrolase family 13 (Henrissat, 1991)), a large group of starch-processing enzymes. The family shares some common characteristics: they act on α-glycosidic bonds, use an α-retaining double displacement mechanism (Koshland, 1953; McCarter and Withers, 1994), contain a $(\beta/\alpha)_8$-barrel catalytic domain and possess four short conserved amino acid sequence regions (Nakajima *et al*., 1986; Svensson, 1994). The reaction and product specificities identified in this family include exo-/endo-specificity, activity towards α-(1,4) or α-(1,6)-glycosidic bonds, preference for hydrolysis or transglycosylation reactions, branching/debranching specificity and even glucan synthesizing activity (Coutinho and Henrissat, 1999; Kuriki and Imanaka, 1999; MacGregor *et al*., 2001). Catalysis in this family proceeds via a covalently bound reaction intermediate (Mosi *et al*., 1997; Uitdehaag *et al*., 1999a), dividing the reactions in two steps: (1) formation of the covalent glycosyl-enzyme intermediate; and (2) transfer of this reaction intermediate to an acceptor molecule. The availability of crystal structures with inhibitors, substrates, products and covalently bound reaction intermediates bound in the active site (mainly CGTases and α-amylases) has provided a detailed insight into the catalytic mechanism of this enzyme family. The details concerning mechanisms of glycoside hydrolases can be found in a number of recent reviews (Rye and Withers, 2000; Uitdehaag *et al*., 2002).

The first structure of an α-amylase family member solved was that of *Aspergillus oryzae* α-amylase (Matsuura *et al*., 1984). Currently (March 2003), 36 α-amylase family enzyme structures have been elucidated (Coutinho & Henrissat, 1999), including five CGTase structures (Harata *et al*., 1996; Klein and Schulz, 1991; Knegtel *et al*., 1996; Kubota *et al*., 1991; Lawson *et al*., 1994). All members possess a $(\beta/\alpha)_8$-barrel catalytic domain (domain A). The loop between β-strand 3 and α-helix 3 of the $(\beta/\alpha)_8$-barrel is rather large and is regarded as a separate domain. This B-domain contributes to substrate binding and is important for reaction specificity, however, neither the fold nor the size of domain B have been conserved (MacGregor *et al*., 2001; Svensson, 1994). The four short conserved amino acid sequence

* Corresponding author. Tel.: + 31-50-3632150. Fax: + 31-50-3632154. E-mail: L.Dijkhuizen@biol.rug.nl

ISSN 1024-2422 print/ISSN 1029-2446 online
DOI: 10.1080/1024242031000161433

regions, which characterize the α-amylase family (Janecek, 1997; Nakajima *et al*., 1986; Svensson, 1994), are located at the C-terminal ends of the β-strands and cluster at the bottom of the $(\beta/\alpha)_8$-barrel. These four conserved sequence regions contain the catalytic nucleophile Asp229, the acid/base catalyst Glu257, and the virtually invariable residues Asp135, His140, Arg227, His327 and Asp328 (amino acid numbering according to *Bacillus circulans* strain 251 (BC251) CGTase). The importance of all these seven conserved residues for catalytic activity has been demonstrated by site-directed mutagenesis (Leemhuis *et al*., 2003a; Libessart and Preiss, 1998; Nakamura *et al*., 1993; Sogaard *et al*., 1993; Strokopytov *et al*., 1995; Vihinen *et al*., 1990). Besides the A and B domains, α-amylase family enzymes are supplemented with variable additional domains. CGTases possess C, D and E-domains C-terminal of the catalytic domain (Klein and Schulz, 1991). The E-domain is a raw starch binding domain (Janecek *et al*., 2003; Ohdan *et al*., 2000; Penninga *et al*., 1996), whereas the functions of domains C and D are less well understood. A few additional domains are found in some other α-amylase family enzymes (e.g. cyclomaltodextrinases and branching enzyme (Abad *et al*., 2002; Park *et al*., 2000)). Thus, the α-amylase family is a diverse group of enzymes that possess a $(\beta/\alpha)_8$-barrel catalytic domain.

CGTase is a unique enzyme of the α-amylase family that catalyzes the formation of circular α-(1,4)-linked oligosaccharides (cyclodextrins) when acting on starch. The cyclodextrins formed consist mainly of six, seven or eight glucose residues (α-, β- and γ-cyclodextrin, respectively), although larger cyclodextrins are formed as well (Penninga *et al*., 1995; Terada *et al*., 1997). Cyclodextrin formation starts with cleavage of the glycosidic bond between the glucose residues bound at subsites $+1$ and -1, resulting in a covalent glycosyl-enzyme intermediate (Mosi *et al*., 1997; Uitdehaag *et al*., 1999a) (Fig. 1). In the second half of the cyclization reaction the nonreducing end of the reaction intermediate moves into acceptor subsite $+1$, followed by intramolecular bond formation to yield a cyclodextrin (Fig. 1). CGTase may also use water or a second sugar molecule as acceptor resulting in a hydrolysis or disproportionation reaction, respectively (Fig. 1). The fourth reaction catalyzed is the coupling reaction, in which a cyclodextrin ring is opened and then transferred to a second sugar molecule to form a linear product (Fig. 1). Interestingly, CGTases have much lower hydrolysis than transglycosylation activities, making the enzyme an efficient transferase (Leemhuis *et al*., 2003b; Penninga *et al*., 1995).

CGTase is used for the industrial production of cyclodextrins, and cyclodextrins are used for their ability to form inclusion complexes with small hydrophobic molecules (e.g. drugs) (van der Maarel *et al*., 2002; van der Veen *et al*., 2000a). However, cyclodextrin yields are limited by product inhibition, incomplete conversion of the starch substrate, lack of cyclodextrin size specificity and hydrolytic activity (van der Veen *et al*., 2000a). As cyclodextrin size specificity depends upon the number of glucose residues that bind at the donor subsites before bond cleavage, this suggests that cyclodextrin size specificity may be modified by altering the substrate binding cleft of CGTases. Thus, structure/function relationship studies could be useful to improve cyclodextrin specificity. However, site-directed mutagenesis studies have not resulted (yet) in mutant CGTases with a strict cyclodextrin size specificity, although this specificity was somewhat altered in many of these mutant enzymes (Kim *et al*., 1997; Lee *et al*., 2002; Leemhuis *et al*., 2002b; Nakamura *et al*., 1994; Parsiegla *et al*., 1998; Penninga *et al*., 1995; Sin *et al*., 1994; van der Veen *et al*., 2000b,c; Wind *et al*., 1998a,b).

HYDROLYSIS AND TRANSGLYCOSYLATION SPECIFICITY

The variation in reaction and product specificity is an intriguing feature of the α-amylase family since the catalytic site architecture is conserved, indicating that specificity is not governed by the catalytic site itself but by nearby sugar binding subsites, by differences in the entrance to the active site, or by induced-fit mechanisms that favor the use of particular substrates. All these phenomena have been described or suggested for α-amylase family enzymes (Kim *et al*., 1999; Kuriki *et al*., 1996; Leemhuis *et al*., 2002b, Leemhuis *et al*., 2003c; Skov *et al*., 2001; Uitdehaag *et al*., 2000; Watanabe *et al*., 1997).

A possible explanation for the differences in hydrolytic and transglycosylation activity of α-amylase family enzymes is variation in the accessibility of the active site to water. However, the active sites of hydrolases as well as transferases in the α-amylase family are rather accessible to solvent, indicating that transglycosylating specificity is not a consequence of shielding the active site from water. Moreover, comparison of the water molecules near the catalytic center of α-amylase family enzymes did not reveal a catalytic water molecule position typical for the hydrolases (Abad *et al*., 2002).

It has been suggested that the hydrophobicity of residues in the vicinity of the catalytic site and, in particular, near the acid/base catalyst are important for the hydrolysis/transglycosylation ratio of α-amylase family enzymes, as mutations in these residues could change this ratio in neopullulanase (Kuriki *et al*., 1996). However, the effects were relatively small (ratios changed by less than 45%)

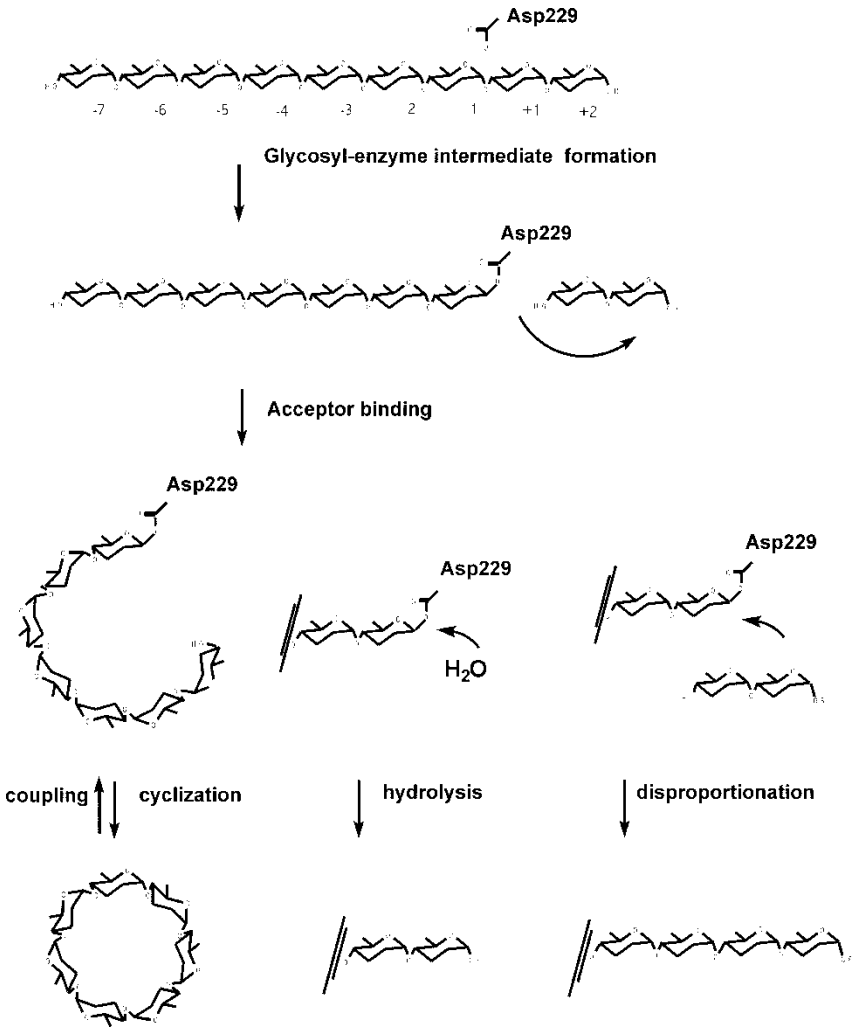

FIGURE 1 The reactions catalyzed by CGTase. Reactions start with cleavage of the α-(1,4)-glycosidic bond between subsites −1 and +1 to form a covalent glycosyl-enzyme intermediate, which is subsequently transferred to an acceptor molecule. The figure has been adapted from Leemhuis *et al*. (2003b).

and the mutations strongly reduced the enzyme activities, which may also explain the altered ratios.

Recently, it has been hypothesized that the separation between the acid/base catalyst Glu257 and Asp328 [a fully conserved α-amylase family residue that stabilizes the transition states during catalysis (Uitdehaag *et al*., 1999a)] may control the hydrolysis/transglycosylation specificity of α-amylase family enzymes (Roujeinikova *et al*., 2001), as this distance is larger in the strict transglycosylating enzymes maltosyltransferase (Roujeinikova *et al*., 2001) and branching enzyme (Abad *et al*., 2002), than in other α-amylase family enzymes. The similar distance between these two residues in the transglycosylase CGTase and the hydrolase α-amylase, however, do not support this hypothesis, or at least show that this is not a general rule.

Another explanation for the variation in hydrolysis and transglycosylation specificity is that glycosyl-enzyme intermediates are favorably stabilized in transferases, which is obviously not necessary in hydrolases, and that a conformational change in the protein (induced by sugar acceptor binding) is required in the second half of a transglycosylation reaction. Structural analysis of CGTase supports this explanation (Uitdehaag *et al*., 2000), which has also been proposed to explain the transglycosylation specificity of branching enzyme (Abad *et al*., 2002). Thus, detailed insights into how α-amylase family enzymes may control their hydrolysis and transglycosylation specificity are gradually emerging.

THE DONOR SUBSITES OF CGTASE ARE IMPORTANT FOR TRANSGLYCOSYLATION SPECIFICITY

Whether CGTase catalyzes hydrolysis or transglycosylation reaction is determined by the nature of the acceptor used, suggesting that reaction specificity is largely controlled by the properties of the acceptor subsites. Nevertheless, mutation studies have shown that the donor subsites of CGTase also contribute to this reaction specificity. Since cyclodextrin size

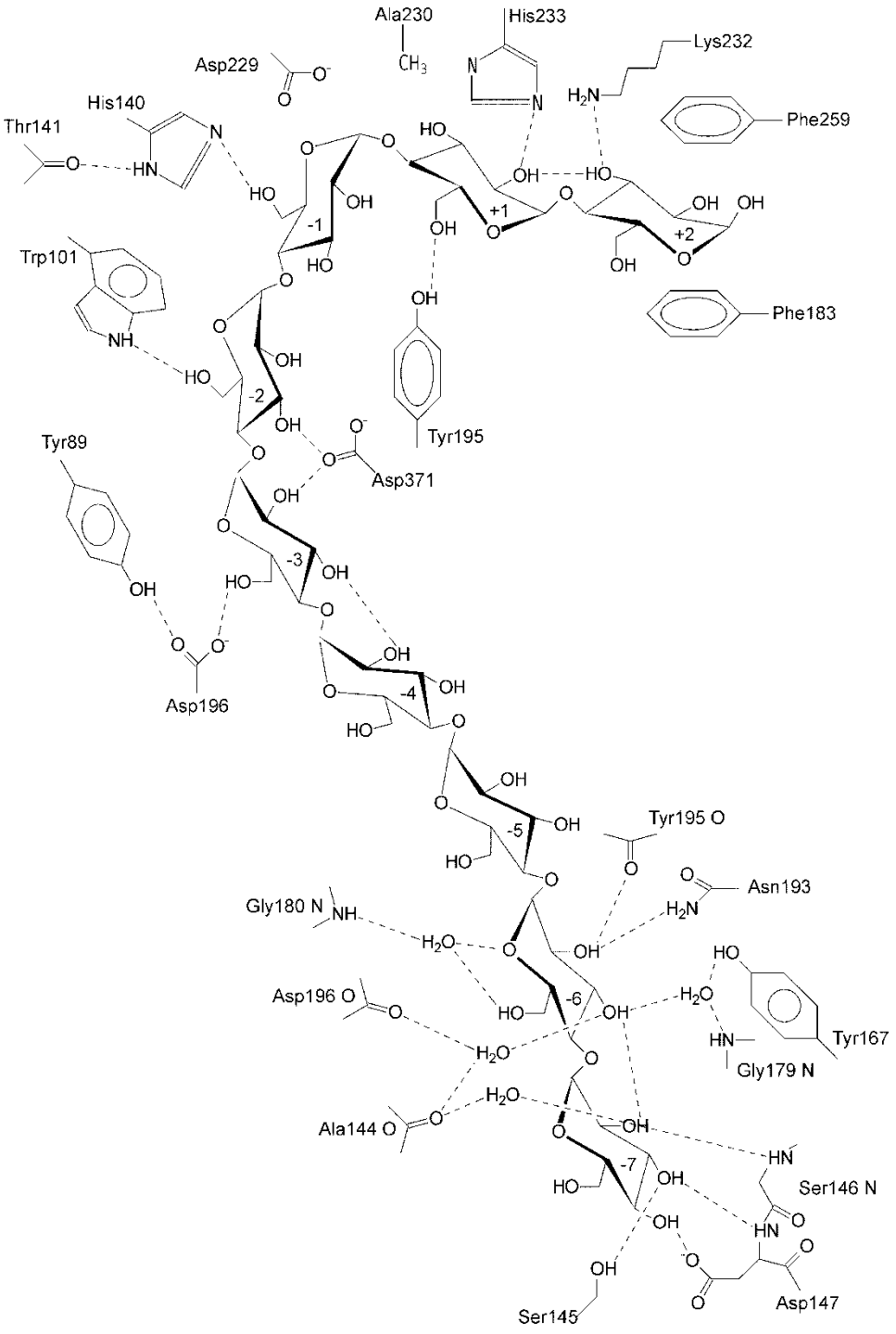

FIGURE 2 Schematic overview of the interactions between BC251 CGTase and a natural maltononaose substrate (Uitdehaag *et al*., 1999a). For clarity, not all interactions at subsites −3, −2 and −1 are shown. The scissile bond is positioned between the subsites −1 and +1, the donor subsites are labeled −1, −2, etc. and the acceptor subsites are labeled +1 and +2 (Davies *et al*., 1997). The figure has been adapted from Uitdehaag *et al*. (1999b).

specificity is determined by the number of glucose residues that bind at the donor subsites prior to glycosyl-enzyme intermediate formation, the donor subsites of CGTases (Fig. 2) have been investigated extensively with site-directed mutagenesis to obtain a better understanding of the cyclization reaction and to be able to control cyclodextrin size specificity. These studies have shown that the donor subsites −1 and −2 are critical for general activity (Kim *et al*., 1997; Mattsson *et al*., 1995; Nakamura *et al*., 1993), that subsite −6 stimulates the use of long substrates in transglycosylation reactions (Leemhuis *et al*., 2002b) and that subsites −3 and −7 are important for cyclodextrin product specificity (Parsiegla *et al*., 1998; van der Veen *et al*., 2000b van der Veen *et al*., 2000c) (the donor subsites −4/−5 have no strong

TABLE I CGTase mutants with reported cyclization and hydrolysis activities. The equivalent position in BC251 CGTase is indicated between brackets

CGTase	Subsite	Cyclization (μmol/min/mg)	Hydrolysis (μmol/min/mg)	Hydrolysis/ cyclization ratio	Ref[c]
Bacillus circulans strain 251					
Wild-type	–	270	3.2	0.012	1
F21L	–	196	10	0.051	2
R47L	−3	70	5.4	0.077	3
R47W	−3	182	15	0.082	2
Y89D	−3	318	4.9	0.015	4
S146P	−7	107	5.9	0.055	4
G179L	−6	215	3.1	0.014	1
G180L	−6	62	3.1	0.050	1
F183N	+2	25	10.6	0.42	5
F183S	+2	15	8.8	0.59	5
N193G	−6	133	3.4	0.026	1
Y195G	Central	143	4.8	0.034	6
A230V	+1	13	72	5.5	2
F259S	+2	42	33	0.79	5
F259N	+2	42	60	1.43	5
F183S/F259N	+2/+2	0.9	14	16	5
F21L/N94S/A230V	−/−3/+1	32	272	8.5	2
Thermoanaerobacterium thermosulfurigenes					
Wild-type		240	54	0.23	7
F184S (183)	+2	52	60	1.1	7
F196G (195)	Central	27	30	1.1	7
D197H (196)	−3	144	17	0.12	8
A231V (230)	+1	10	138	14	2
F260N (259)	+2	31	117	3.8	7
F260L (259)	+2	33	174	5.3	7
F260I (259)	+2	17	174	10.2	7
F260E (259)	+2	19	177	9.3	7
F284K (283)	–	86	8	0.09	8
N327D (326)	–	51	18	0.35	8
F184S/F260N (183/259)	+2	5	118	24	7
F196G/F260N (195/259)	Central/+2	0.8	15	19	7
TDPAG insertion	−3/−4	0.8	13.8	17	9
Bacillus stearothermophilus strain ET1					
Wild-type		193[a]	46[a]	0.24[a]	10
F191G (195)	Central	27[a]	21[a]	0.78[a]	10
F255I (259)	+2	19[a]	101[a]	5.3[a]	10
Bacillus stearothermophilus strain NO2					
Wild-type		7.6[b]	1.9	0.25	11
F255I (259)	+2	< 0.01[b]	4.3	-	11
W254V (260)	–	1.7[b]	1.9	1.12	11

[a] Arbitrary activity units. These hydrolysis/cyclization ratios cannot be compared with those of other CGTases.
[b] α-Cyclization activity, β- and γ-cyclodextrin forming activities were not described.
[c] References: 1, (Leemhuis *et al*., 2002b); 2, (Leemhuis *et al*., 2003d); 3, (van der Veen *et al*., 2000b); 4, (van der Veen *et al*., 2000c); 5, (van der Veen *et al*., 2001); 6, (Penninga *et al*., 1995); 7, (Leemhuis *et al*., 2002a); 8, (Wind *et al*., 1998b); 9, (Leemhuis *et al*., 2003a); 10, (Lee *et al*., 2002); 11, (Fujiwara *et al*., 1992).

interactions with substrate; Fig. 2). In addition, these studies have demonstrated that the donor subsites contribute to the transglycosylation specificity, as most of these mutants lowered the transglycosylation specificity of CGTase, as indicated by the increased hydrolysis/cyclization ratio (Table I). However, these effects were rather small and did not (strongly) enhance the hydrolytic activity. Thus, all donor substrate binding subsites typical for CGTase (subsites −3, −6 and −7) contribute to the unique characteristic of the enzyme, transglycosylation specificity and cyclodextrin formation.

A comparison of the *Bacillus stearothermophilus* maltogenic α-amylase (product name Novamyl from Novozymes) and CGTases provides another example of the role of donor subsites in reaction specificity. These enzymes have a very similar three-dimensional structure (94% of C_α atoms with a rmsd of 1.1 Å) (Dauter *et al*., 1999), but Novamyl is a maltose producing starch hydrolase (Christophersen *et al*., 1998). This different reaction specificity is caused by a loop extension in Novamyl that hampers the binding of substrates of sufficient length to form a cyclodextrin product (Fig. 3). This was demonstrated by removing the loop extension (five amino acid residues), which changed Novamyl into a cyclodextrin-producing enzyme (Beier *et al*., 2000). The opposite experiment, introducing the loop extension into CGTase, converted CGTase into a starch hydrolase (Table I) (Leemhuis *et al*., 2003d), albeit with a low activity. These results also show the importance of donor substrate binding subsites for

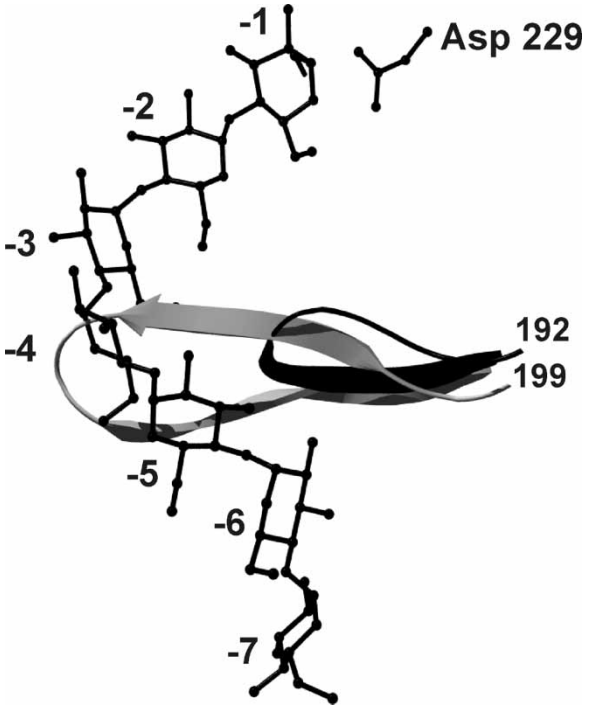

FIGURE 3 Superposition of *T. thermosulfurigenes* CGTase [black; protein data bank (Berman *et al*., 2000) code 1CIU (Knegtel *et al*., 1996)] and the maltogenic α-amylase from *B. stearothermophilus* [gray; PDB code 1QHO (Dauter *et al*., 1999)]. For clarity only the five residue loop extension of the maltogenic α-amylase plus four flanking residues at both sides, the corresponding loop of CGTase and the catalytic nucleophile (Asp230) are shown. The sugar shown (black) in the donor subsites −7 to −1 of *T. thermosulfurigenes* CGTase is modeled using the maltononaose bound BC251 CGTase structure as template (Uitdehaag *et al*., 1999a). The figure has been adapted from Leemhuis *et al*. (2003d). This figure and Fig. 4 were made with SwissPdbViewer (Guex and Peitsch, 1997).

hydrolysis and transglycosylation specificity of CGTase.

THE ACCEPTOR SUBSITES CONTROL THE REACTION SPECIFICITY OF CGTASE

The nature of the acceptor substrate, binding at the acceptor subsites, largely determines whether a hydrolysis or transglycosylation is catalyzed. The preference of CGTase for sugar acceptor substrates is most likely determined by the properties of the acceptor subsites. Interestingly, the acceptor subsite +1 architecture of CGTase is very similar to that of α-amylases (Brzozowski and Davies, 1997; Klein and Schulz, 1991; Lawson *et al*., 1994; Matsuura *et al*., 1984; Qian *et al*., 1994), whereas the architecture of acceptor subsite +2 is typical for and conserved in CGTase (Klein and Schulz, 1991; Lawson *et al*., 1994; Strokopytov *et al*., 1996). This suggests that the transglycosylation specificity of CGTase is governed by subsite +2, and not by subsite +1. Subsite +1 is, however, important for the general enzyme activity of CGTases and α-amylases (Nakamura *et al*., 1993; Nielsen *et al*., 1999; Sogaard *et al*., 1993). The residues at subsite +2 (Fig. 2) are also important for general enzyme activity (Matsui *et al*., 1992; Nakamura *et al*., 1994; van der Veen *et al*., 2001), and in addition they (Phe183 and Phe259) are determinants of the reaction specificity of CGTase (Fujiwara *et al*., 1992; Lee *et al*., 2002; Leemhuis *et al*., 2002a; Nakamura *et al*., 1994; van der Veen *et al*., 2001).

Besides decreasing the transglycosylation activities (e.g. cyclization), several of the mutations in Phe183 and Phe259 strongly enhanced the low hydrolytic activity of CGTase, mutations of Phe259 especially (Table I). Thus, the hydrolysis/cyclization ratio could be strongly increased by mutations at subsite +2 (Table I). Double mutants of Phe183/Phe259 had, however, no higher hydrolytic activities, although the hydrolysis/cyclization ratio was further enhanced (Table I). As the mutations in Phe183 and Phe259 lowered the hydrophobicity of acceptor subsite +2, it was suggested that the hydrophobic nature of subsite +2 may limit the hydrolytic activity of CGTases (van der Veen *et al*., 2001). However, replacement of Phe259 by hydrophobic (Ile/Leu) and hydrophilic (Asn/Glu) residues with similar sized side chains increased the hydrolytic activity and the hydrolysis/cyclization ratio of *Thermoanaerobacterium thermosulfurigenes* CGTase to a similar extent (Table I), demonstrating that neither the hydrolytic activity nor the hydrolysis/cyclization ratio were limited by the hydrophobicity of the side chain at position 259. Thus, the aromatic side chain of Phe259 is instrumental in limiting the hydrolytic activity of CGTase and mutations of Phe259 can change CGTase into an efficient starch hydrolase.

Recently, BC251 CGTase was subjected to random mutagenesis in a search for CGTase mutants displaying increased hydrolytic activity to identify amino acid residues crucial for the transglycosylation specificity of the enzyme. The most interesting mutation identified (A230V) reduced the cyclization activity 20-fold, while it enhanced the hydrolytic activity 24-fold, converting CGTase into a starch hydrolase (Table I). This Ala230 residue is located at acceptor subsite +1 (Fig. 2) and is conserved in CGTase. Although Ala230 is part of conserved sequence region II of the α-amylase family (Janecek, 1997; Nakajima *et al*., 1986; Svensson, 1994), this alanine residue is not conserved throughout the α-amylase family; α-amylases, for example, contain an alanine, serine, threonine or phenylalanine residue at the equivalent position. Mutation of the corresponding alanine residue in *Bacillus stearothermophilus* α-amylase to serine and glycine residues reduced the activity by 98% (Nielsen *et al*., 1999). Saturation mutagenesis of Ala230 in BC251 CGTase showed that most mutations at this position drastically

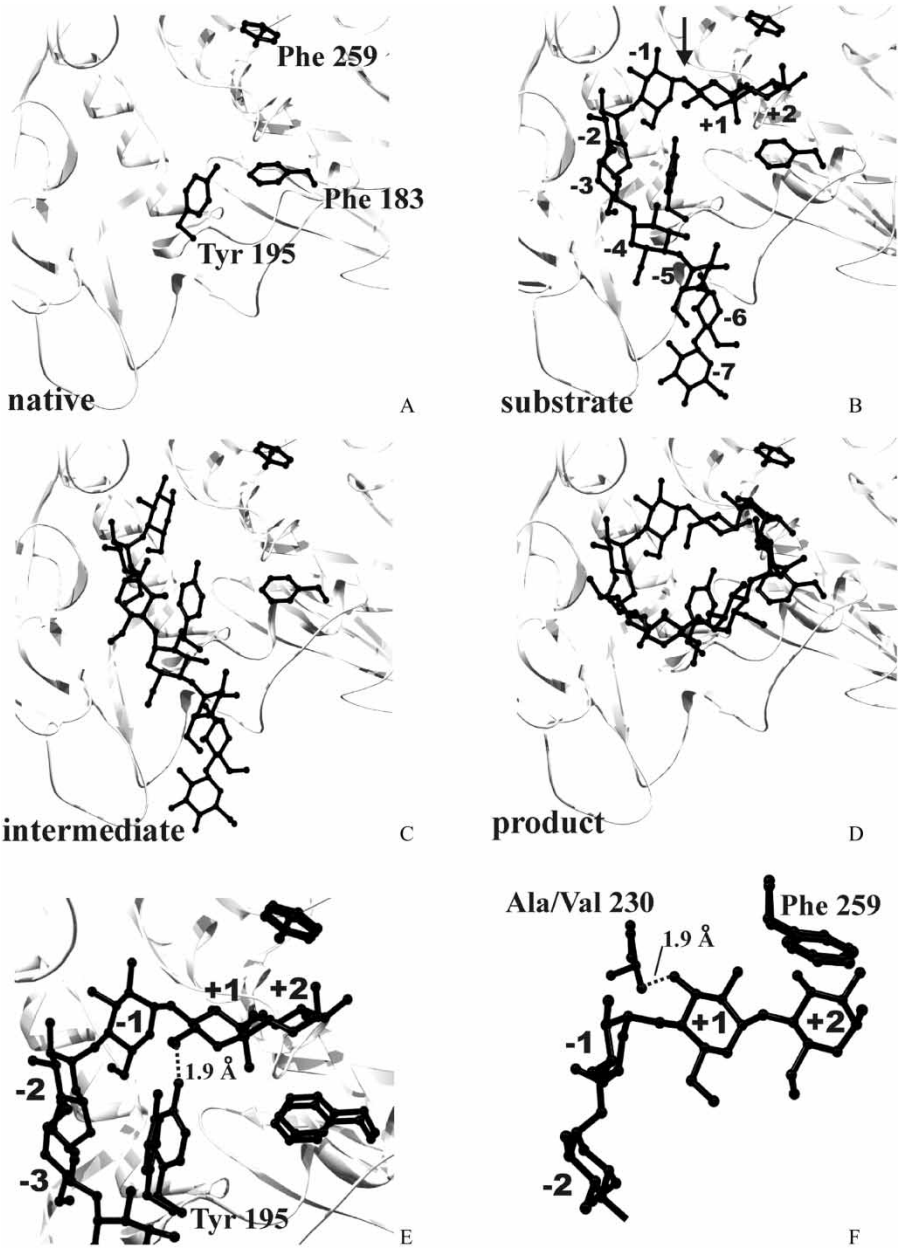

FIGURE 4 Close-up views of the active site of BC251 CGTase structures along the reaction pathway of cyclodextrin formation (A – D). Native CGTase (A), substrate-CGTase complex (B), maltoheptaose reaction intermediate bound CGTase (C), γ-cyclodextrin product bound CGTase (D). Panel E shows an overlay of the substrate and reaction intermediate bound structures. Panel F shows a superposition of the substrate bound CGTase structure with the structure of mutant A230V. The views in the panels A – E are from the same direction, whereas panel F is a different view to show the Ala/Val230 side chains. Selected amino acids and sugars are shown in black and the protein backbone in gray. The arrow in panel B indicates the cleavage site. The dashed lines (panels E and F) show the close-contacts between Tyr195 and Val230 with the glucose in subsite + 1 when the sugar would bind in same manner as in the maltononaose bound structure. The PDB codes used are 1CDG (Lawson *et al*., 1994), 1CXK (Uitdehaag *et al*., 1999a), 1EO5 (Uitdehaag *et al*., 2000), 1D3C (Uitdehaag *et al*., 1999b) and the A230V structure (Leemhuis *et al*., 2003c).

reduced the cyclization activity and that only the change to a valine residue increased the hydrolytic activity significantly (Leemhuis *et al*., 2003c). Thus, an A230V mutation converts CGTase into a starch hydrolase.

The random mutagenesis study also showed that the hydrolytic activity of mutant A230V could be further increased by additional rounds of mutagenesis and selection. Three rounds yielded a mutant CGTase (F21L/N94S/A230V) with the highest hydrolytic activity ever reported for a CGTase, at a level similar to the cyclization activity of the wild-type CGTase (Table I).

The identification of mutation A230V was unexpected, as Ala230 is located at subsite + 1, which has a rather similar architecture in the hydrolase α-amylase and the transglycosylase CGTase (Brzozowski and Davies, 1997; Klein and Schulz, 1991; Lawson *et al*., 1994; Matsuura *et al*., 1984; Qian *et al*., 1994) and because all subsite + 1 mutants described

before had strongly reduced activities (Nakamura *et al*., 1993; Nielsen *et al*., 1999; Sogaard *et al*., 1993). Mutant A230V had indeed reduced overall activities (Leemhuis *et al*., 2003c), which may be explained by the effect of the larger valine side chain on the initial substrate binding. However, the high hydrolytic activity of this mutant demonstrates that the glycosyl-enzyme intermediate is still formed at a significant rate, but that water has become the preferred acceptor substrate. This indicates that the A230V mutation hampers the use of sugar acceptors, whereas the formation of the glycosyl-enzyme intermediate is less affected. An explanation for this is that the orientation of the glucose in subsite +1 is more critical for bond formation than for bond cleavage (Leemhuis *et al*., 2003c). Whether the increased hydrolytic activity is caused by more productive binding of water in the active site of the mutant or that the mutation destabilizes the glycosyl-enzyme intermediate is unknown. Thus Ala230 at acceptor subsite +1 is crucial for the transglycosylation specificity of CGTase.

AN INDUCED-FIT MECHANISM MAY EXPLAIN THE TRANSGLYCOSYLATION SPECIFICITY OF CGTASE

For CGTase, it has been argued that the transglycosylation activity of the enzyme is enhanced via an induced-fit mechanism upon binding of an acceptor sugar in acceptor subsite +1 in the second half of the reaction (Uitdehaag *et al*., 2000). This hypothesis is based on structural work, which showed that acceptor sugar binding at acceptor subsite +1 is hampered when a maltoheptaose is bound in the donor subsites −7 to −1 only, but not in native, substrate (in subsites −7 to +2) or product bound CGTase structures (Uitdehaag *et al*., 2000). This maltoheptaose bound CGTase structure represents the reaction intermediate with a sugar in the donor subsites and empty acceptor subsites. Fig. 4 (panels A–D) shows the different CGTase structures along the reaction pathway. In the maltoheptaose bound structure, Tyr195 has a conformation that hinders acceptor sugar binding at subsite +1 (the hydroxyl group of Tyr195 will form a close contact with the glucose in subsite +1; Fig. 4E), indicating that acceptor sugar binding changes the conformation of Tyr195, which in turn induces structural rearrangements (in an unknown manner) that activate the enzyme in transfer of the reaction intermediate to the acceptor. The glucose residues bound at subsites −6, −3, and +1 play a key role in this induced-fit activation (Uitdehaag *et al*., 2000) and sugar binding at subsite −6 has been shown to activate the enzyme in transglycosylation reactions (Leemhuis *et al*., 2002b). Since water is a much smaller acceptor molecule, water binding at subsite +1 will not induce the structural rearrangements necessary to activate the enzyme in catalysis, explaining the low hydrolytic activity and thus the transglycosylation specificity of CGTase (Uitdehaag *et al*., 2000).

The strongly decreased k_{cat} values and the unaffected, or even lower, K_M values for acceptor substrates in the disproportionation reaction of acceptor subsite mutants [in Phe183, Lys232, His233 and Phe259 (Nakamura *et al.*, 1993, 1994; van der Veen *et al*., 2001)] are consistent with the induced-fit mechanism proposed. These kinetic parameters indicate that these acceptor subsite residues are important for the catalytic efficiency especially, and less critical for the acceptor binding. It seems likely that these residues are important for the orientation of the incoming sugar acceptor, in a way that is optimal for catalysis of a transglycosylation reaction.

The much higher hydrolytic than cyclization and disproportionation activities of mutant A230V (Table I) (Leemhuis *et al*., 2003c) show that the covalent glycosyl-enzyme intermediate is still formed at a significant rate (at least 72 μmol/min · mg, or a k_{cat} of 90 s^{-1}) and that this intermediate is preferably transferred to water. As the A230V mutation most likely hinders acceptor sugar binding at subsite +1 (Fig. 4F), this mutation is thought to interfere with the proposed induced-fit activation mechanism, resulting in a strongly decreased transglycosylation. Thus, the drastically reduced transglycosylation activities of mutant A230V provide biochemical evidence for the occurrence of an induced-fit activation mechanism in the second step of transglycosylation reactions, as the glycosyl-enzyme intermediate is still formed at a high rate.

CONCLUSIONS

Structural and biochemical work have yielded a detailed insight in the catalytic machinery of CGTase. However, the transglycosylation specificity is far from understood. How does CGTase select sugar acceptor substrates specifically and what causes the low hydrolytic activity of CGTase? Mutation studies have demonstrated that the hydrolysis/transglycosylation ratio of CGTase can be altered by mutations at the donor and acceptor substrate binding subsites. However, only mutations at acceptor subsites +1 and +2 can change CGTase into an efficient starch hydrolase. Thus, the architecture of acceptor subsites +1 and +2 is responsible for the low hydrolytic activity of CGTase. Moreover, insights are gradually emerging that the transglycosylation specificity of CGTase is the result of an induced-fit mechanism that stimulates the use of sugar acceptors.

Acknowledgements

H.L. thanks Danisco for financial support.

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